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Histopatholqgical Response to Turpentine in the White Shrimp, *Penaeus setiferus*[1]

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Observations are presented on the inflammatory response to a highly irritative substance, turpentine, injected into the abdominal musculature of the white shrimp, *Penaeus setiferus*. Injections of the irritant were administered with a tuberculin syringe between the fifth and sixth segments. Penaeid shrimp were found to be highly sensitive to turpentine, even when administered in small dosages. When sterile petroleum jelly was mixed with the turpentine to reduce the dispersion rate, the shrimp's "internal defense mechanism" was able to combat effectively the effect of the irritant. Postinjection observations of the tissues at the site of injection, gill, heart, and hepatopancreas were made at 8, 16, 24, 32, 40, 48, 72, 96, 120, 168, and 240 hr, and at 15, 20, 30, 40, 50, 60, and 120 days. The induced cellular inflammatory response consisted of infiltrating hemocytes and fibrocytes resulting in the formation of fibrous capsules, brown melanized nodules, and fibrous scar tissue in all tissues examined. The gills and hepatopancreas showed considerable tissue destruction early, but were eventually cleared of the histopathological effects of the turpentine and later appeared normal. However, extensive tissue destruction was easily distinguishable in the heart and abdominal muscle even at 120 days postinjection.

INTRODUCTION

The inflammatory response in invertebrates as well as vertebrates is a synergistic complex of humoral and cellular responses. This internal defense mechanism has been studied extensively in the vertebrate animal, and is correlated to the phylogenetic level of the various species. Little is known, however, of the inflammatory response in invertebrates, particularly of the humoral responses. What is known about the cellular phase of the inflammatory response in invertebrates other than insects has been reviewed by Bang (1970) and Sparks (1972).

Recently, histopathology of marine decapod crustacea, particularly penaeid shrimp, has received considerable attention with respect to potential problems with diseases in mariculture situations. A basic understanding of the inflammatory response of penaeid shrimp is useful to a program of prophylaxis and treatment of disease in these animals.

The wound repair processes in penaeid shrimp (Fontaine and Lightner, 1973) and development of scar tissue after wounding (Fontaine and Dyjak, 1973) have been reported. Observations on the phagocytosis and elimination of a foreign abiotic particulate material injected into white shrimp, *Penaeus setiferus,* have been recorded (Fontaine and Lightner, 1974). Evidence of hemocytic activity in penaeid shrimp was noted in a fungal disease (Lightner and Fontaine, 1973), a tumor (Sparks and Lightner, 1973), and in the development of a fibrous capsule (unpublished).

Turpentine, a highly irritative substance, has been used to invoke the inflammatory response in the Pacific oyster, *Crassostrea gigas* (Pauley and Sparks, 1965, 1966), as well as the rainbow trout, *Salmo gairdneri irideus* (Weinreb, 1958, 1959). Observations on the inflammatory response to turpentine injected into the abdominal musculature of the white shrimp are presented in this paper.

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TABLE 1
Results of Turpentine Tolerance Tests on the White Shrimp, *Penaeus setiferus*

Experiment No.	Number of animals	Base media	Concentration of turpentine % by volume	Volume injected (ml.)	Turpentine injected	Percent mortality	Duration total time
1	10	none	100	0.10	0.10	100	3 min
2	10	none	100	0.05	0.05	100	3 min
3	10	none	100	0.02	0.02	100	3 min
4	50	mineral oil	5	0.05	0.003	74	24 hr
5	50	vaseline	10	0.10	0.01	10	24 hr
6	50	vaseline	10	0.05	0.005	8	24 hr
7	50	vaseline	10	0.02	0.002	2	24 hr
8	50	vaseline	5	0.01	0.005	8	24 hr
9	50	vaseline	5	0.05	0.003	8	24 hr
10	50	vaseline	5	0.02	0.001	2	24 hr

Materials and Methods

The shrimp used in this study were purchased from a local bait dealer and were, according to his description, captured with a trawl from along the Houston ship channel in Galveston Bay, Texas. These shrimp, ranging in total length from 90 to 120 mm, were allowed to acclimate to laboratory conditions for a period of 72 hr. Injured or obviously unhealthy animals were removed.

Test shrimp and a group of untreated controls were held in a 2000-liter tank provided with an undergravel filter. Control animals were distinguished by the absence of fluorescent pigment granules that were injected into test animals. Each day the shrimp were examined, fed, and all dead animals removed. Temperatures ranged from 21° to 30°C and the salinities from 22 to 29‰ during the study.

Tolerance tests showed that white shrimp were extremely sensitive to turpentine (Table 1). As little as 0.02 ml of undiluted commercial grade turpentine administered by intramuscular injection between the fifth and sixth abdominal segments resulted in 100% mortality of test animals within 3 min of injection. The injected shrimp were hyperactive and exhibited loss of equilibrium within 1 min, and were immobile on their sides a few seconds later. The heart beat was irregular after 2 min, and had ceased by 3 min postinjection. In an attempt to localize the irritant and minimize the dispersion rates, turpentine was mixed with either sterile petroleum jelly or sterile mineral oil. Results of tolerance tests using pure turpentine and petroleum jelly–turpentine mixtures are given in Table 1.

Test animals for histological study were given intramuscularly 0.05-ml sterile petroleum jelly containing 10% turpentine by volume between the fifth and sixth abdominal segments with a 1-ml syringe. In addition, the fluorescent pigment Dayglo Neon Red[3] was mixed with the petroleum jelly–turpentine solution to allow for visual tracing of the dispersion of the mixture in the body of the shrimp. Observations of the fluorescent particles in living specimens were made in a darkened room using ultraviolet light.

Samples from two shrimp were taken for histological examination at 8, 16, 24, 32, 40, 48, 72, 96, 120, 168, and 240 hr, and at 15, 20, 30, 40, 50, 60, and 120 days postinjection. The fifth segment and the cephalothorax were taken from each specimen and fixed in 10% phosphate-buffered formalin. Tissue specimens were prepared for light microscopy according to standard histological procedures. The fifth segment was sectioned sagittally and the cephalothorax was cross sectioned. All tissues were embedded in

[3]The use of trade names in this publication does not imply endorsement of commercial products.

paraffin, sectioned at 8–10 μm, and stained with hematoxylin and eosin.

The gross morphological nomenclature used here is from Young (1959).

Results

Gross Observations

The fluorescent pigment entered the lateral artery between the fourth and fifth abdominal somites at the site of injection (8 hr), moved to the subneural artery, from the subneural artery to the median sternal sinus, and into the gills (16 hr). From the gills, the pigment moved through the heart (24 hr) and out the dorsal abdominal artery through the posterior arterial connective (30 hr) and back into the subneural artery (48 hr). This accounts for fluorescent pigment observed in the sixth abdominal somite and in the telson. It was assumed that dispersion of the petroleum jelly–turpentine mixture was the same as that visualized by pigment dispersion.

Histological Observations

Site of injection. Necrosis of muscle in the area of injection was apparent within 8 hr postinjection. Large foci of amorphous eosinophilic cellular debris appeared in the abdominal muscle tissue by 72 hr, evidently a result of focal necrosis. Around the periphery of these necrotic areas, the muscle bundles were infiltrated by hemocytes and fibrocytes; many of these infiltrating cells had pyknotic nuclei. Associated with the fibrocytes were many collagenlike fibers (Fig. 1). Much of the eosinophilic cellular debris in the interstitial spaces was probably a result of cytolysis of the infiltrating hemocytes.

The areas of focal myonecrosis had been effectively encapsulated by hemocytes and fibrocytes by 240 hr postinjection (Fig. 2). These large fibrous structures were prevalent in the ventral abdominal muscle tissue just basal to the epidermal epithelium. They persisted throughout the study and in later

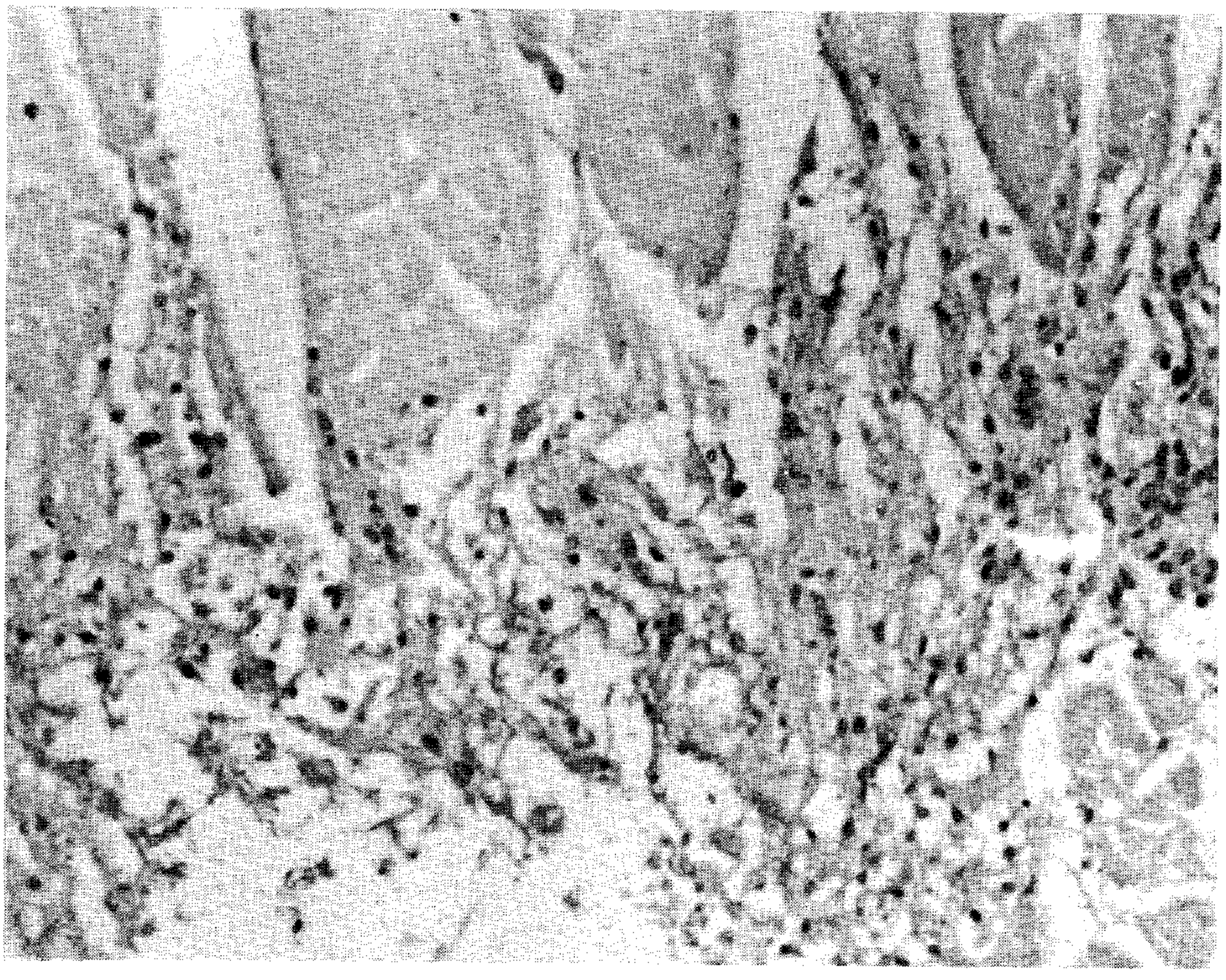

FIG. 1. Infiltration of hemocytes and fibrous tissue near the site of injection, 72 hr. H & E., × 280.

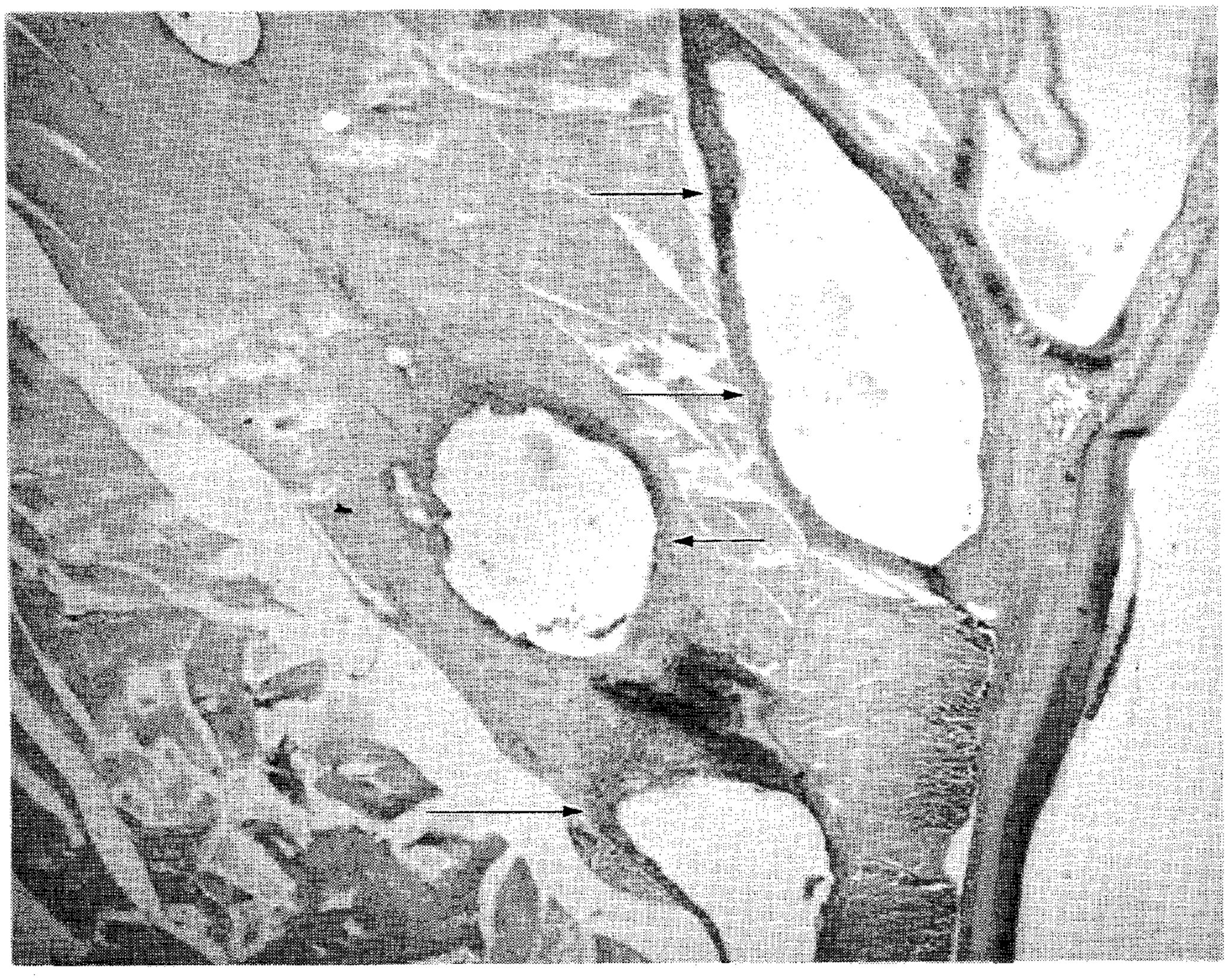

FIG. 2. Large abscesslike structures observed at 240 hr postinjection. H & E, × 70.

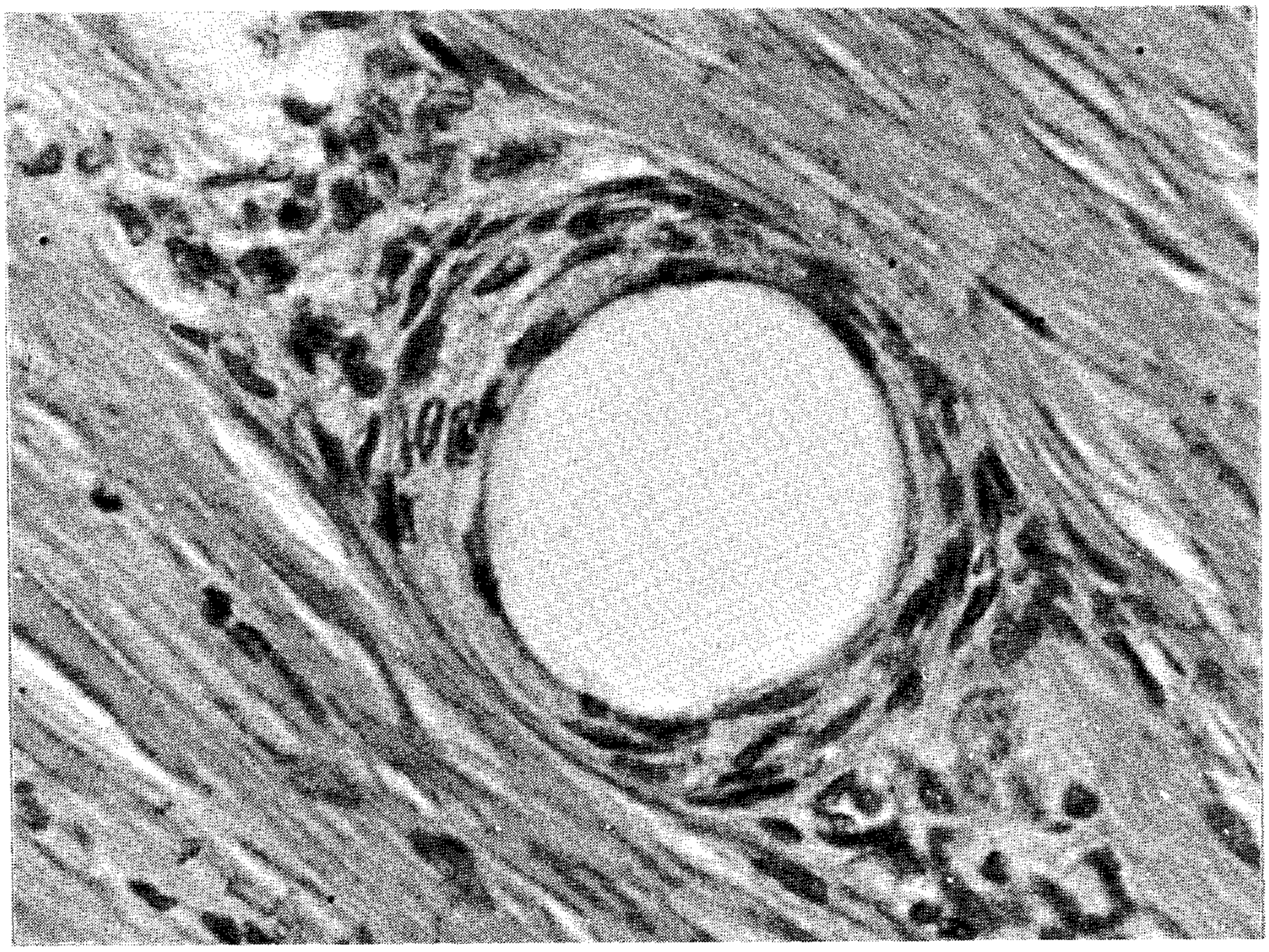

FIG. 3. The large abscesslike structures which persisted throughout the study, 120 days postinjection. H & E, × 700.

stages were reduced in size and appeared in section as rounded vacant capsules (Fig. 3). These large fibrous cysts were peculiar in that, unlike cellular inflammatory responses observed previously in penaeid shrimp (Fontaine and Lightner, 1974), little, if any, melanization of accumulated hemocytes occurred.

Other isolated incidences of tissue destruction near the site of injection were observed in some samples. The midgut from one animal had undergone necrosis at 24 hr postinjection (Fig. 4). The ventral nerve ganglion in another test animal at 8 hr was necrotic, infiltrated by hemocytes, and had numerous foci of encapsulation appearing.

Gills. Many areas of the gill tissue were necrotic by 8 hr postinjection. The lumens of many gill lamellae were congested with hemocytes, many with pyknotic nuclei. Within 24 hr, numerous melanized nodules were formed in the gills (Fig. 5). Formation of these nodules was the result of hemocyte activity in the necrotic gill tissue and such nodules were observed in all test shrimp through 60 days postinjection. However, the incidence of nodules decreased with time. Although fluorescent pigment granules were in the lumen of the gill filaments at 120 days, the gills were clear of melanized nodules and the tissue had a normal histologic appearance. The clearing of the gills was probably due to gradual sloughing of the brown "scablike" nodules, including the encapsulated material, with the molt.

The relative number of peritrichous protozoans associated with the gill filaments increased beginning at 24 hr postinjection reaching a peak at 10 days in a manner similar to that observed in the oyster (Pauley and Sparks, 1965, 1966). An increase in peritrichous protozoans has also been observed in normal postmortem change of the gill tissue in penaeid shrimp (Lightner, 1974). The increase in the numbers of ciliates associated with the gills may indicate a reduction in the normal activity of the shrimp, particularly with regard to respiratory processes.

Tegumental glands are normal his-

FIG. 4. Necrosis of the midgut at 24 hr (G). The abdominal muscle ventral to the midgut is necrotic and consists of only a hyaline mass (M). H & E, × 440.

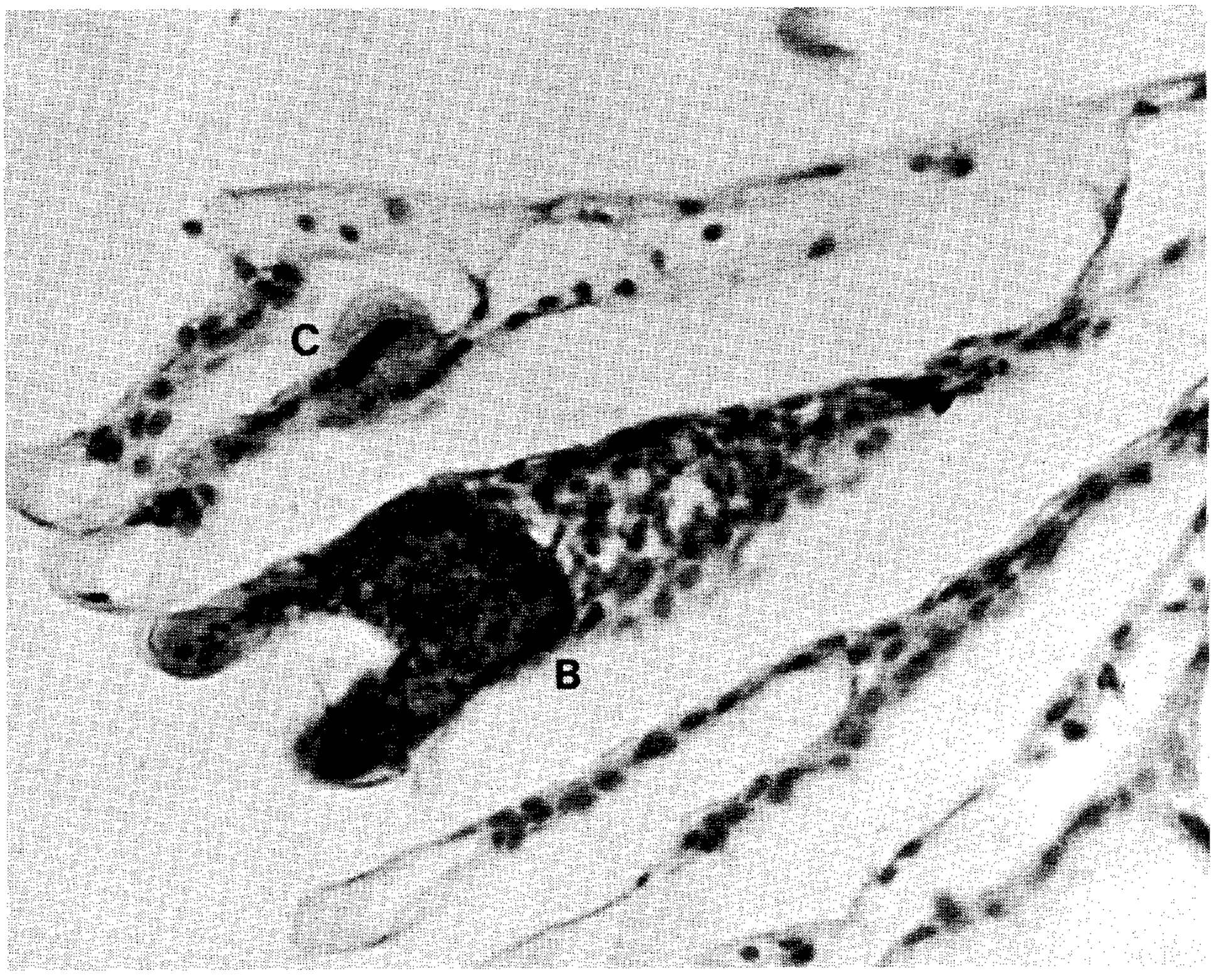

FIG. 5. Melanized nodules (B) in the gills at 24 hr postinjection (C = peritrichous protozoan). H & E, × 440.

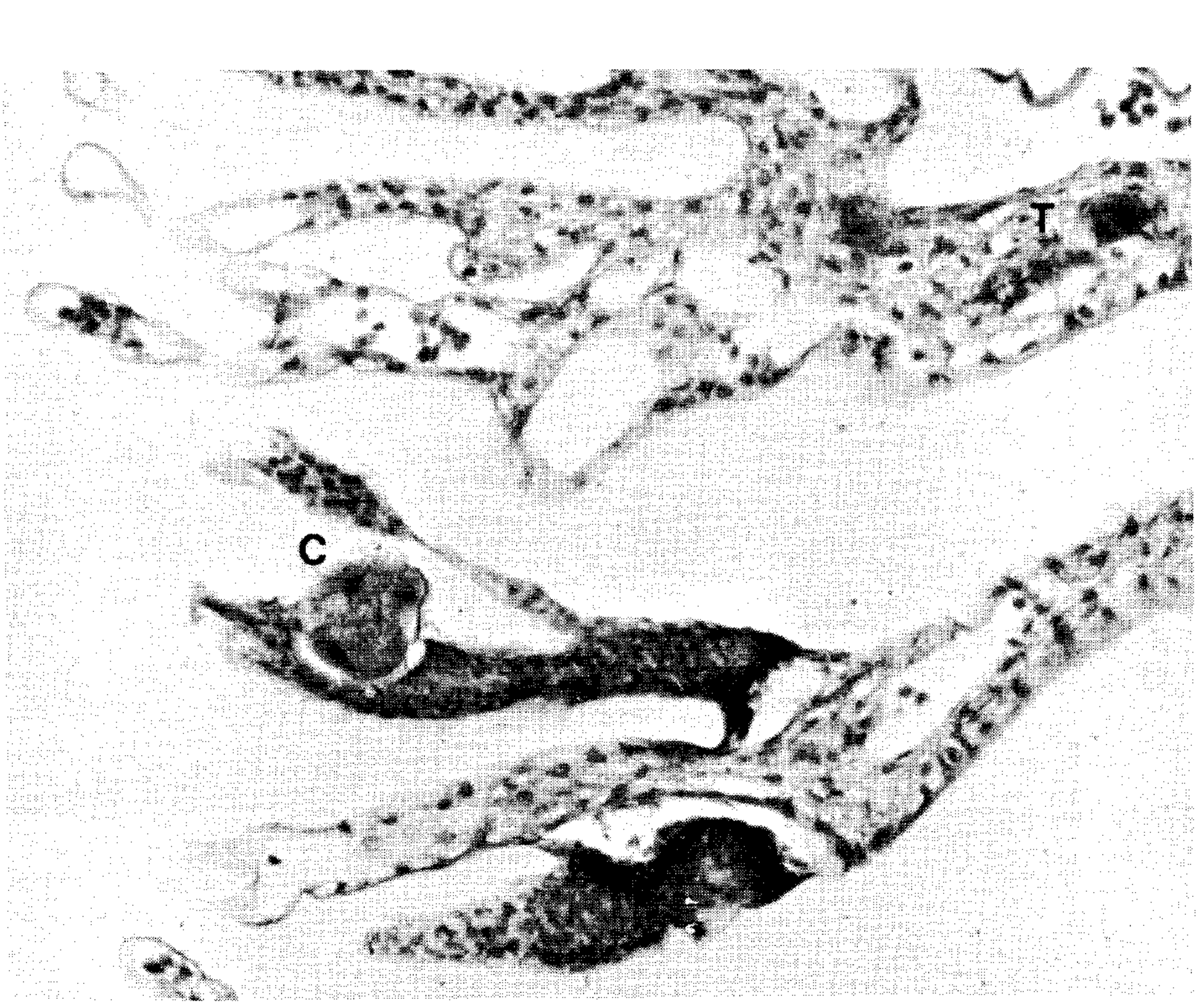

FIG. 6. Hypertrophied and hyperchromatic tegumental glands of the gill at 72 hr (T = gland; C = peritrichous protozoan). H & E, × 280.

tological structures in the gill tissue of shrimp, but these are not numerous and are much smaller than those associated with the external body cuticle. At 24 hr postinjection a proliferation of the tegumental glands of the gills was noted, and by 72 hr these glands were hypertrophied and hyperchromatic (Fig. 6). The numbers of tegumental glands decreased with time after 240 hr; however, their former locations may have been represented by clusters of large basophilic cells (Fig. 7) that persisted throughout the remainder of the study in the gills just basal to the epidermis.

Heart. The tissue that appeared affected most severely by turpentine circulating in the hemolymph was the myocardial fibers. At 8 hr postinjection the nuclei of many of the myocardial fibers were pyknotic (Fig. 8); within 24 hr melanized nodules had appeared. These structures were composed of hemocytes and represented an acute inflammatory response within the heart.

At 240 hr the heart was laden with hemocytes and fibrocytes. Many collagen-like fibers also had appeared and formed, replacing myocardial fibers with dense scar tissue. Interspersed in the scar tissue were numerous melanized nodules, probably formed in response to the necrotic muscle tissue (Fig. 9). The myocardial fibers were dissociated, hypertrophied, and most nuclei were pyknotic.

The extent of the inflammatory response within the heart varied throughout the study from virtually no response to an intense response. The degree of myonecrosis in some specimens, particularly at 240 hr, and the resulting fibrillar and capsular formation within the heart, indicated a condition approaching complete cardiac disfunction. It was amazing to the authors that shrimp showing the degree of tissue destruction just described survived this chemical trauma and upon sampling appeared grossly normal and exhibited normal behavior.

Hepatopancreas. The rapid autolytic changes postmortem of the hepatopancreas

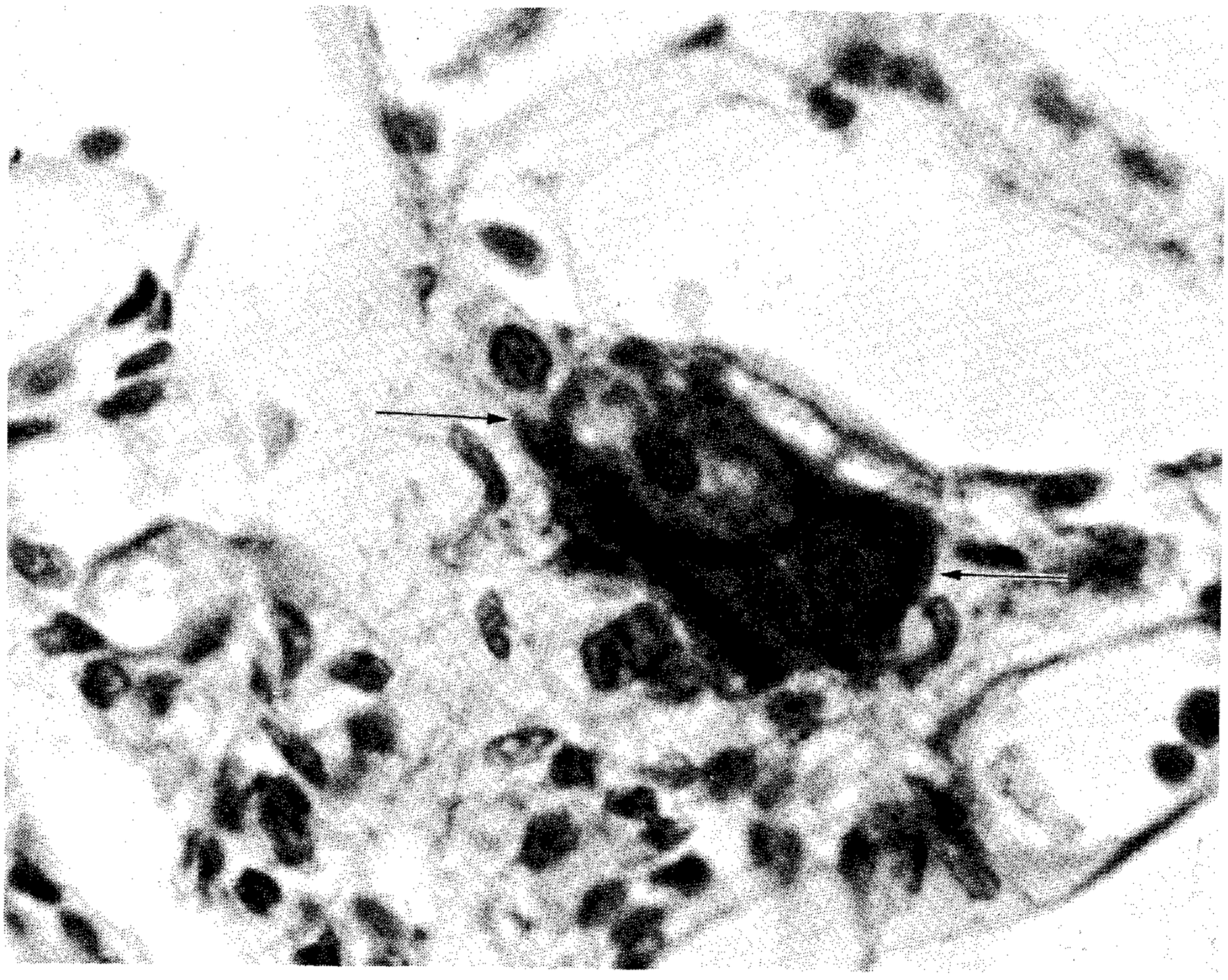

FIG. 7. Cluster of large basophilic cells which appeared by 10 days and persisted throughout the study in the gills just basal to the epidermis. H & E, × 1100.

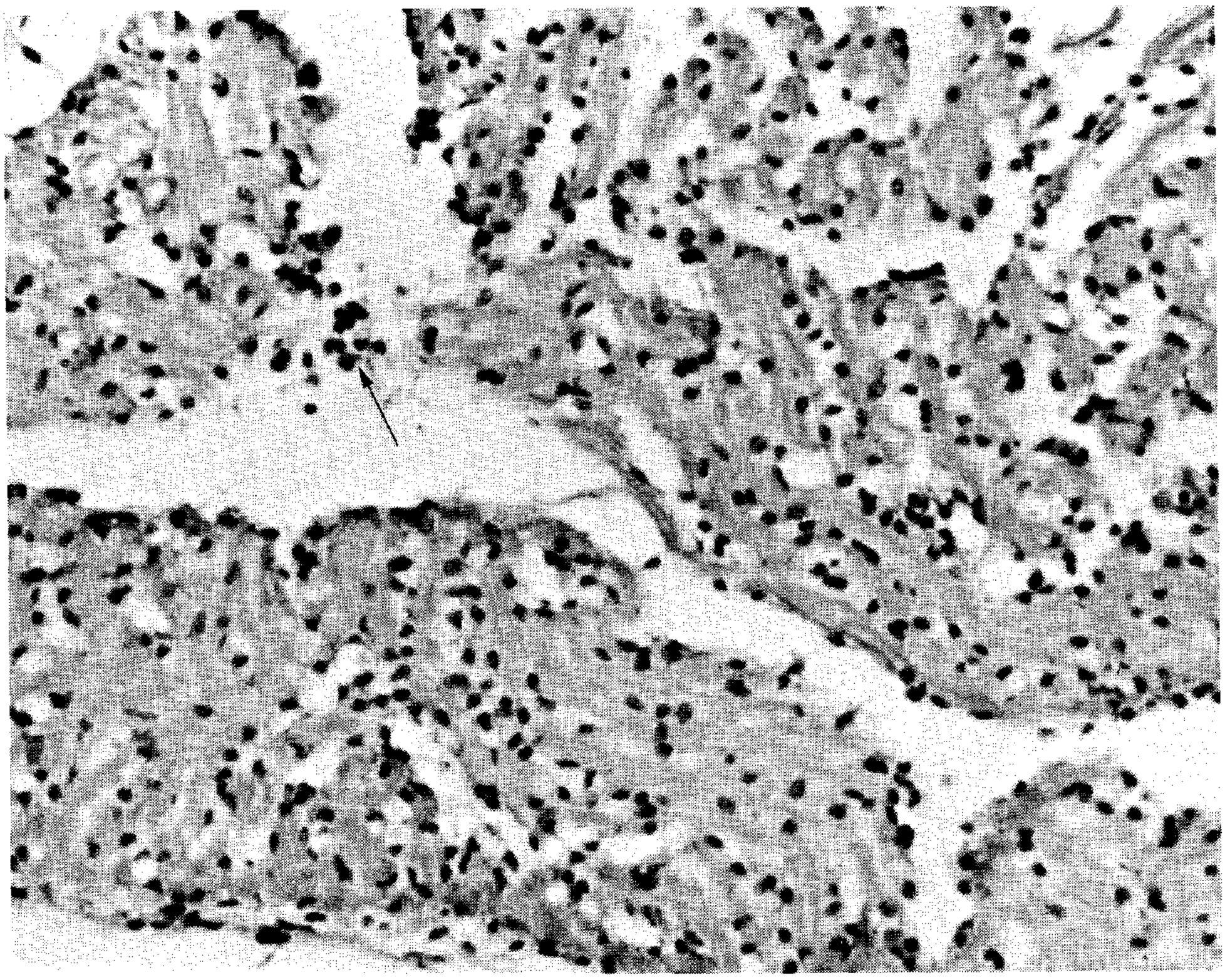

FIG. 8. Pyknotic nuclei of myocardial muscle fibers with necrotic hemocytes in the blood sinuses (arrow), 8 hr. H & E, × 280.

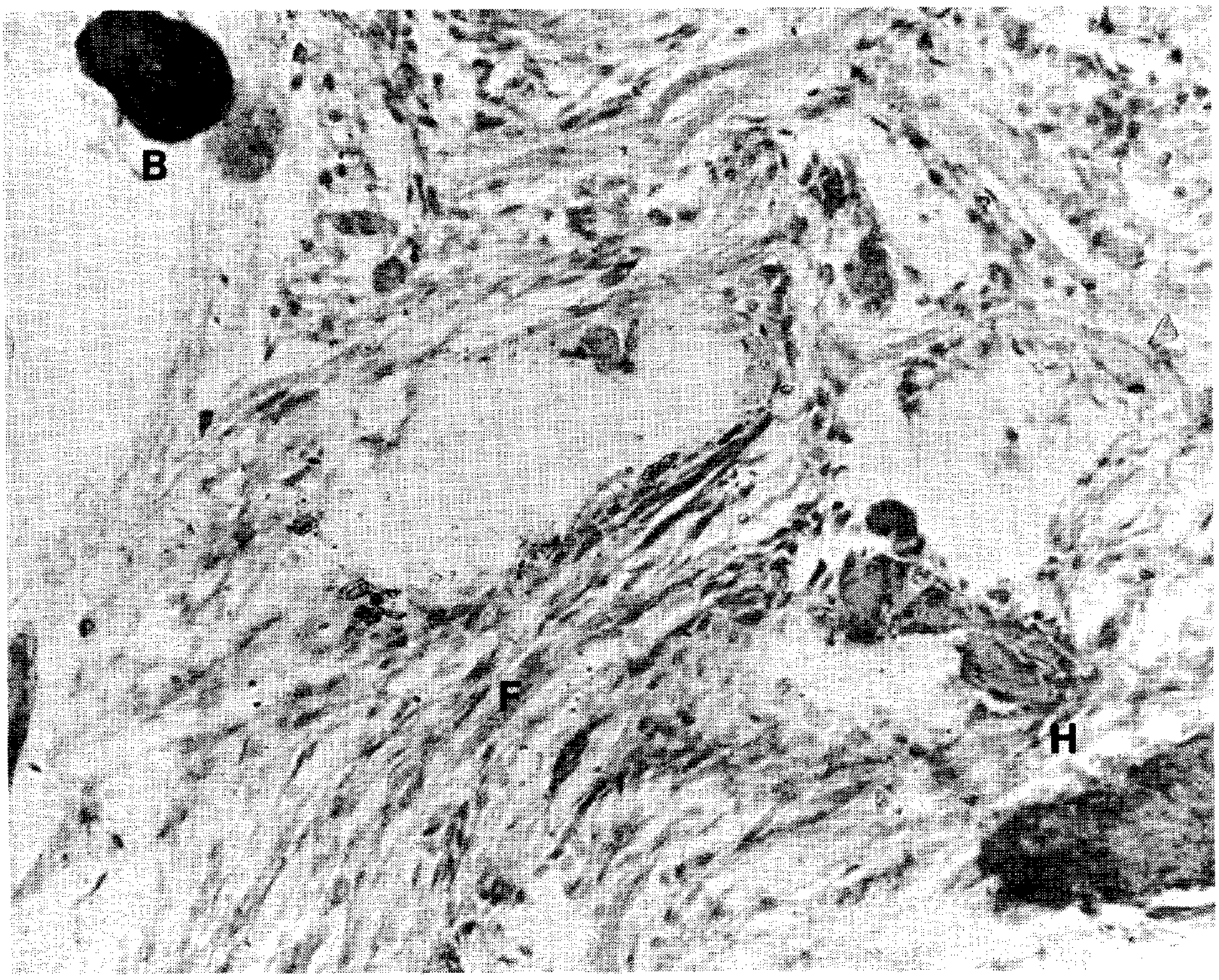

FIG. 9. Heart at 240 hr congested with hemocytes and fibrocytes. Many collagenlike fibers (F) form a dense granulative scar tissue throughout. Interspersed in the tissue are numerous melanized nodules (B) and melanizing foci of hemocytic encapsulation (H). H & E, × 700.

in penaeid shrimp (Lightner, 1974; Sparks and Fontaine, 1973) make it difficult to discern normal histologic change post-mortem from histologic change induced by the irritant. Within 8 hr postinjection the hepatopancreas of shrimp injected with turpentine was congested with hemocytes, many of which were necrotic. By 24 hr the digestive tubule epithelium of the hepatopancreas was highly vacuolated. This vacuolation could have been a result of poor fixation, but there were numerous melanized nodules and congestion of intertubular spaces with hemocytes indicating necrosis of the hepatopancreatic tissue rather than normal autolysis. The greatest intensity of the inflammatory response was at 240 hr and consisted of melanized nodules (Fig. 10) and congestion of hemocytes in the digestive tubule interstices. The inflammatory response within the hepatopancreas decreased with time. By 120 days postinjection the hepatopancreas appeared normal with the exception of several small melanized nodules marking the foci of previous hemocytic encapsulations.

Discussion

Shrimp injected with undiluted turpentine in this study survived no longer than 3 min postinjection. Petroleum jelly mixed with the turpentine apparently reduced the dispersion rate so that the histopathological effects of the irritant were both acute and chronic.

According to Pauley and Sparks (1966), oysters that were injected with 0.05 ml of pure turpentine may possess the ability to combat successfully a toxic substance such as turpentine. However, if turpentine gains entry to the bloodstream, it may be spread throughout the oyster's body, causing widespread necrosis of vital tissues, which may eventually result in the death of the oyster although the latter did not occur during the time limits of their investigation. The extent of the necrosis of vital tissues in shrimp injected with a turpentine–petroleum jelly mixture and the inability of shrimp to tolerate even small injections of pure turpentine may be explained, in part, by the anatomical difference in the circulatory system of the two animals.

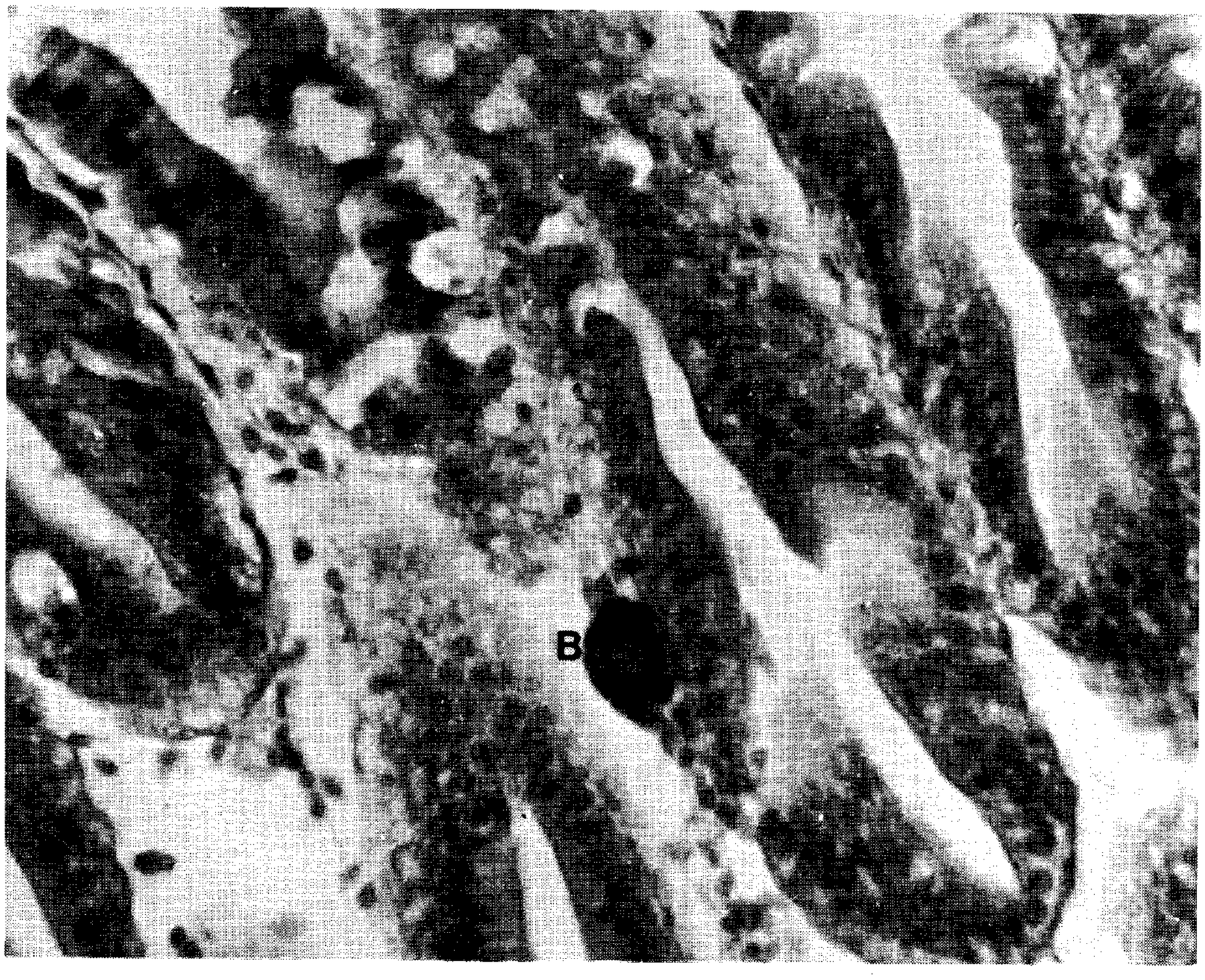

Fig. 10. A melanized nodule (B) in the hepatopancreas at 240 hr postinjection. H & E, × 440.

Histologically, the cellular responses in the oyster and in the shrimp are generally the same with one notable exception. The encapsulations formed around the necrotic tissue or foreign material (petroleum jelly and pigment granules) in shrimp were thick fibrous capsules with many fibrocytes in association with the hemocytes, while cysts formed in the oyster were not fibrous.

Responses of digestive tract tissues were studied over a 72-hr period following turpentine injection in the rainbow trout, *Salmo gairdneri irideus* (Weinreb, 1959). The cellular inflammatory responses, as with the oyster, were generally comparable to that of the shrimp. There was an increase in the number of fibrocytes and granular cells in the stomach after 7 hr followed by a decrease in granule cell numbers at 24 hr. In the trout the digestive tract had returned to "almost normal" after 2 days. This differs strikingly from the shrimp in that histological changes were recognizable 120 days postinjection.

References

Bang, F. B. 1970. Disease mechanisms in crustacean and marine arthropods. *In* "A Symposium on Diseases of Fishes and Shellfishes" (S. F. Snieszko, ed.), pp. 383–404. Amer. Fish. Soc. Spec. Publ. No. 5. Washington, DC.

Fontaine, C. T. and R. G. Dyjak. 1973. The development of scar tissue in the brown shrimp, *Penaeus aztecus,* after wounding with the Petersen disk tag. *J. Invertebr. Pathol.,* **22,** 476.

Fontaine, C. T. and Lightner, D. V. 1973. Observations on the process of wound repair in penaeid shrimp. *J. Invertebr. Pathol.,* **22,** 23–33.

Fontaine, C. T. and Lightner, D. V. 1974. Observations in the phagocytosis and elimination of carmine particles injected into the abdominal musculature of the white shrimp, *Penaeus setiferus. J. Invertebr. Pathol.,* **24,** 141–148.

Lightner, D. V. 1974. Normal postmortem changes in the brown shrimp, *Penaeus aztecus* Ives. *Fish. Bull.,* **72,** 223–236.

Lightner, D. V. and Fontaine, C. T. 1973. A new fungus disease of the white shrimp, *Penaeus setiferus. J. Invertebr. Pathol.,* **22,** 94–99.

Pauley, G. B. and Sparks, A. K. 1965. Preliminary observations on the acute inflammatory reaction in the Pacific oyster, *Crassostrea gigas* (Thunberg). *J. Invertebr. Pathol.,* **7,** 248–256.

Pauley, G. B. and Sparks, A. K. 1966. The acute inflammatory reaction in two different tissues of the Pacific oyster, *Crassostrea gigas. J. Fish. Res. Bd. Can.,* **23,** 1913–1921.

Sparks, A. K. 1972. "Invertebrate Pathology: Noncommunicable Diseases." Academic Press, New York/London.

Sparks, A. K. and Fontaine, C. T. 1973. Host response in the white shrimp (*Penaeus setiferus*) to infection by the larval trypanorhynchid cestode, *Prochristianella penaei. J. Invertebr. Pathol.,* **22,** 213–219.

Sparks, A. K. and Lightner, D. V. 1973. A tumorlike papilliform growth in the brown shrimp (*Penaeus aztecus*). *J. Invertebr. Pathol.,* **22,** 203–212.

Weinreb, E. L. 1958. Studies on the histology and histopathology of the rainbow trout, *Salmo gairdneri irideus.* I. Hematology: under normal and experimental conditions of inflammation. *Zoologica,* **43,** 145–154.

Weinreb, E. L. 1959. Studies on the histology and histopathology of the rainbow trout, *Salmo gairdneri irideus.* II. Effects of induced inflammation and cortisone treatment on the digestive organs. *Zoologica,* **44,** 45–53.

Young, J. H. 1959. Morphology of the white shrimp, *Penaeus setiferus* (Linnaeus, 1758). *Fish. Bull.,* **59,** 1–168.